

B<sub>12</sub> binding proteins that circulate in the blood. In each instance, these increases in TC transport proteins and the corresponding systemic depletion of B<sub>12</sub> were not the result of megaloblastosis, granulocyte proliferation, or any other pathogenic B<sub>12</sub> deficiency.

In a second example of receptor-mediated endocytosis, folate receptors that mediate endocytotic activity have previously been identified in bacterial cells (Kumar et al., 1987) and used for delivery of biologically active materials (Low et al., 1995). Folic acid, folinic acid, pteropolylglutamic acid, and folate receptor-binding pteridines such as tetrahydropterins, dihydrofolates, tetrahydrofolates and their deaza and dideaza analogs are useful as targeting molecules in accordance with the present invention. The terms "deaza" and "dideaza" analogs refer to the art-recognized analogs having a carbon atom substituted for one or two nitrogen atoms in the naturally-occurring folic acid structure. For example, the deaza analogs include the 1-deaza, 3-deaza, 5-deaza, 8-deaza, and 10-deaza analogs. The dideaza analogs include, for example, 1,5-dideaza, 5,10-dideaza, 8,10-dideaza, and 5,8-dideaza analogs. The foregoing folic acid derivatives are conventionally termed "folates," reflecting their capacity to bind with folate-receptors, and such ligands when complexed with exogenous molecules are effective to enhance trans-membrane transport. Other folates useful as complex forming ligands for this invention are the folate receptor binding analogs aminopterin, amethopterin (methotrexate), N<sup>10</sup>-methylfolate, 2-deamino-hydroxyfolate, deaza analogs such as 1-deazamethopterin or 3-deazamethopterin, and 3',5'-dichloro-4-amino-4-deoxy-N<sup>10</sup>-methylpteroyl-glutamic acid (dichloromethotrexate). Other suitable ligands capable of binding to folate receptors to initiate receptor-mediated endocytotic transport of the complex include anti-idiotypic antibodies to the folate receptor. An exogenous molecule in complex with an anti-idiotypic antibody to a folate receptor is used to trigger trans-membrane transport of the complex. Such molecules are used in accordance with the present invention as a targeting molecule.

In a further example of receptor-mediated endocytosis, biotin receptors have been used to mediate endocytotic activity (Low et al., 1995). Biotin analogs such as biocytin, biotin sulfoxide, oxybiotin and other biotin receptor-binding compounds are ligands that may also be used as suitable targeting molecules to promote the trans-membrane transport of exogenous molecules in accordance with this invention. Other compounds capable of binding to biotin receptors to initiate receptor-mediated endocytotic transport of the complex are also contemplated. These can include other receptor-binding ligands such as, for example, anti-idiotypic

antibodies to the biotin receptor. An exogenous molecule complexed with an anti-idiotypic antibody to a biotin receptor could be used to trigger trans-membrane transport of the complex. Such molecules are used in accordance with the present invention as a targeting molecule.

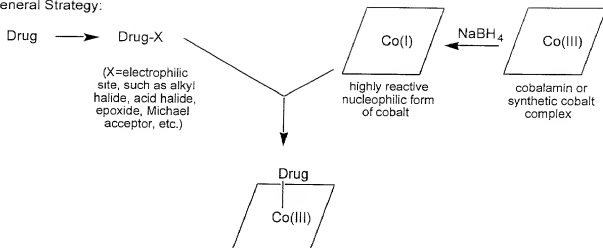
Other examples of targeting molecules include glucose, galactose, mannose, mannose 6-phosphate, hormones (e.g., insulin, growth hormone, and the like), growth factors or cytokines (e.g., TGF- $\beta$ , EGF, insulin-like growth factor, and the like), YEE(GalNAcAH)<sub>3</sub> or derivatives, cobalamin,  $\alpha$ -2 macroglobulins, asialoglycoprotein, albumin, texaphyrin, metallotexaphyrin, antibodies, antibody fragments (e.g., Fab), single-chain antibody variable region (scFv), transferrin, any vitamin and any coenzyme.

As previously described, a bioconjugate of the present invention comprises a bioactive agent conjugated directly or indirectly via a covalent bond to the cobalt atom of an organocobalt complex. The bioactive agent is conjugated directly to the cobalt atom through a non-reactive atom in the bioactive agent or is conjugated indirectly to the cobalt atom through the use of a spacer. Therefore, in contrast to the conjugates formed under U.S. Patent 5,428,023, the attachment of a bioactive agent to the cobalt atom in the axial position does not interfere with receptor-mediated endocytosis from the blood into cells.

The unusually weak cobalt-non-reactive atom bond (e.g., C-Co bond) of the bioconjugate provides a readily addressable trigger for the controlled *in vivo* release of the bioactive agent from the organocobalt complex. The bond dissociation energy (BDE) of Co-non-reactive atom bond in the bioconjugate is in the range of 30 to 50 kcal/mol (e.g., 30-40 kcal/mol range for a Co-C bond) which make them among the weakest covalent bonds known, yet the bond is relatively stable in aqueous solution.

A common strategy will employ the modification of the anticancer drug so that it possesses an electrophilic site which can react with the highly nucleophilic Co(I) intermediate generated upon treatment of hydroxycobalamin with NaBH<sub>4</sub>. This structural modification will be sufficiently far removed from the active site (pharmacophore) to preclude any interference with the desired biological activity. Approaches used in the case of chlorambucil are typical: the carboxylic acid group of chlorambucil is converted to either an acid chloride or a bromoethyl ester, either of which can be efficiently coupled with cob(I)alamin.

General Strategy:



For example, reduced  $\text{Cbl}^{\text{I}}$  is prepared by  $\text{NaBH}_4$  or zinc dust reduction, e.g. of hydroxocob(III)alamin. In the above scheme, the drug can be a cytotoxic agent, other drug or other bioactive agent as described herein. In other schemes, a spacer containing a carbon atom or other atom such as that specified for the non-reactive atom for binding to the cobalt atom and which also contains a reactive grouping, e.g.  $-\text{OH}$  or  $-\text{CN}$ , which is further reacted with the bioactive agent, is introduced. Other reactive groups, e.g.  $-\text{NH}_2$ ,  $-\text{SH}$ ,  $-\text{COOH}$ , etc., can also be utilized for coupling to a bioactive agent. It is important to note that, in some cases (e.g., chlorambucil, doxorubicin), the small organic molecule released is not the parent drug, but rather retains some of the modification installed to allow coupling. In other cases (e.g., topotecan), the structure of the released drug may correspond to the parent molecule.

More specific details of the synthesis of representative bioconjugates according to the present invention are as follows, using a "drug" which can be replaced by any suitable bioactive agent and cobalamin which can be replaced by any suitable organocobalt complex. In this synthesis, all procedures are under argon. Hydroxocob(III)alamin is dissolved in aqueous  $\text{CH}_3\text{OH}$  (1:1 v/v) at  $25^\circ\text{C}$ . A 2-10 fold excess of  $\text{NaBH}_4$  is added. The solution slowly changes color from red to brown and gradually green ( $\text{Cbl}^{\text{I}}$ ). After approximately 15 min. the electrophilic drug ligand (dissolved in the same deoxygenated solvent) is added, e.g., as an alkyl, acyl or aryl chloride. Strictly anaerobic conditions are maintained and the reaction mixture is stirred gently at  $25^\circ\text{C}$ . The color gradually changes back to red as  $\text{Cbl}^{\text{I}}$  is converted to alkyl-, acyl-, or aryl- $\text{Cbl}^{\text{III}}$ . After about 1.5 h, the solution is acidified to pH 3.0 with dilute  $\text{HCl}$ . Methanol is removed under reduced pressure by rotary evaporation at less than  $40^\circ\text{C}$ . The